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# Real-Time Salmonella Detection Using Lead Zirconate Titanate-Titanium Microcantilevers

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## ABSTRACT

Current methods for analysis of unknown powders in suspicious packages involve sending samples to laboratory facilities where a variety of time-consuming tests are performed. We have developed and investigated the use of a lead zirconate titanate - titanium (PZT-Ti) microcantilever for *in situ* detection of the common food- and water-born pathogen, *Salmonella typhimurium*. Using a bifunctional linking molecule to immobilize antibody on the titanium surface of the microcantilever, we can directly detect salmonella cells in suspensions of differing concentration. This novel surface functionalization technique along with the sub-nanogram sensitivity of the cantilever has allowed for direct quantification of *S. typhimurium* cells in suspension.

## INTRODUCTION

Salmonella is a water-born/food-born pathogen that causes more than five hundred deaths each year [1]. It is also a category B bioterrorism agent that can seriously contaminate water resources. There is no device at present that can actually monitor a space and perform an analysis of the air or water within that space in real-time. However, the presence of such a device would dramatically decrease the "lag time between release of an agent and its detection," and thus enable more prompt treatment of those individuals possibly exposed to a particular bioterrorism agent in addition to reducing the total number of individuals affected [2].

The current methods employed for analyzing bacterial pathogens include filtration and subsequent cell colony growth and identification, polymerase chain reaction (PCR) [3], and Enzyme-Linked Immunosorbent Assay (ELISA) [4]. The method of filtration and cell culture (which involves the collection of biological agents by passing the sample through a filter medium and growth of colonies in the filter medium for 12 hours to 2 days) is the current definite method for determining the presence of a bacterial pathogen [3]. With PCR, cells must be lysed to release the DNA which is amplified for detection. The ELISA method requires fluorescent labeling for optical detection. These methods are tedious, time consuming and neither *in situ* nor quantitative.

In addition to the above methods, surface plasmon resonance (SPR) and the quartz crystal microbalance (QCM) are also used for probing biological interactions and the presence of biological agents. SPR detects molecular binding on a surface by measuring the refractive index change due to such binding [5]. QCM detects mass changes by measuring the thickness-mode resonance frequency shift of the quartz crystal [6]. Both SPR and QCM are capable of *in situ* analysis as well as quantification, but they do not readily lend themselves to the development of an array type sensor needed for efficient and cost-effective production of systems for monitoring of public spaces. The silicon microcantilever has the advantage of high detection sensitivity but it cannot withstand the damping caused by water [7]. The Q value which is the ratio of the

resonance peak frequency to the peak width at half the peak height drops from as high as 100 in air to less than 1 in water, rendering silicon microcantilevers not suitable for aqueous detection.

The piezoelectric microcantilever developed by our group, [8,9] on the other hand, can maintain strong resonance peaks in water with Q value well over 40 by optimally incorporating a highly piezoelectric layer in the cantilever structure, thus allowing direct, in-water detection, while also presenting advantages such as highly sensitive, *in situ*, real-time quantification and low cost of production. This work is the basis for the microcantilevers used herein to directly detect the presence of *Salmonella typhimurium* in aqueous suspension. Our detection of salmonella in aqueous suspension suffers not from the issue of damping, nor does it require fluorescent labeling; yet it maintains the ability to be developed into an array detection system. In addition, the piezoelectric microcantilever uses electric means for both actuation and detection, the sensor and its measuring unit are small, light-weight, and can be easily portable.

## MATERIALS AND METHODS

These microcantilevers were fabricated by bonding a layer of lead zirconate titanate (PZT) (T105-H4E-602, Piezo Systems, Inc., Cambridge, Massachusetts) 0.127 mm thick, 2 mm long, and 1.5 mm wide to a titanium foil (Alfa Aesar, Ward Hill, MA) 0.127 mm thick with a titanium tip 3 mm long (see Fig. 1). The PZT layer had a piezoelectric coefficient $d_{31}$ of -320 pm/V and a Young's modulus $Y_{11}$ of 62.5 GPa. The titanium foil has a Young's Modulus of 103 GPa. When excited by an alternating current, the cantilever vibrates due to the piezoelectric effect. The resonance frequencies depend on the physical properties and dimensions of the cantilever, as well as the properties of the surrounding medium [9]. These resonant frequencies can be identified and monitored by measuring the electrical impedance spectrum with an impedance analyzer (Agilent 4294A, Agilent, Palo Alto, CA). The resonant peaks occur where the phase angle (a ratio of the real part of impedance to its imaginary part) is a maximum. Binding of antigens or cells to the cantilever tip increases the cantilever mass which in turn decreases the cantilever's resonance frequency. Monitoring the shift in frequency of a resonant peak, $\Delta f_r$, can be translated into the amount of mass, $\Delta m$, adhered to the tip under detection conditions by the following equation [9]:

$$\Delta m \approx -\left(\frac{2M_e}{f_r}\right)\Delta f_r. \tag{1}$$

where $f_r$ and $M_e$ denote the resonance frequency and the effective mass of the cantilever, respectively. Figure 1, presents an image of the actual cantilever used along with a schematic of
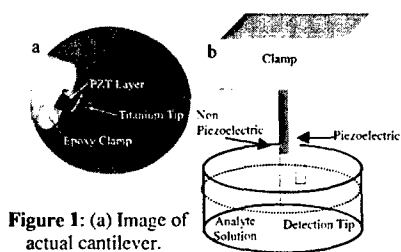


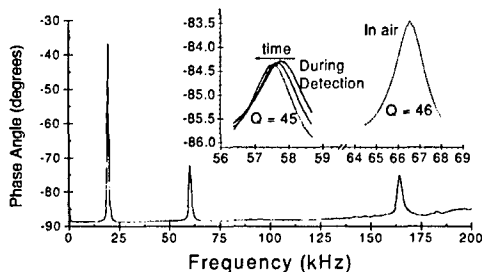**Figure 1**: (a) Image of actual cantilever. (b) Schematic of cantilever detection in aqueous solution.

**Figure 2**: Phase Angle versus Frequency spectrum of PZT-Ti cantilever with detection peaks inset

the detection setup while Figure 2 is a sample resonance spectrum from the PZT-Ti cantilever and the inset shows one particular resonant peak in air along with the same peak when the cantilever tip was submerged in a cell suspension during detection. It can be seen that as time elapsed during detection, the resonant peak position shifted to lower frequencies. This was due to the adhesion of analyte to the sensor surface and it is this principle that allows for these microcantilevers to be used for precise analyte quantification.

In this study, a biological antibody-antigen system is used to demonstrate the ability of such cantilevers to detect and quantify biological agents *in situ* and in real-time. The antigen used herein was the salmonella strain *Salmonella typhimurium* and the antibody used was an antibody to Salmonella Common Structural Antigens (CSA-1) (both from Krikegaard & Perry Laboratories, Inc., Gaithersburg, Maryland). This antibody is specifically designed to recognize several species of bacteria in the *Salmonella* genera, including our *typhimurium* species. In order to detect the presence of the salmonella bacteria in buffer suspension, the CSA-1 antibody was immobilized on the tip of a PZT-Ti microcantilever by means of a linking molecule. Titanium was chosen as the non-piezoelectric layer as a result of its good mechanical properties as well as its outstanding biocompatibility [10]. This biocompatibility is demonstrated by its widespread use as a material for implants in the human body. And although we are detecting antigens that are harmful to the humans, we do need to ensure that the surface does not otherwise harm or repel our analyte cells.

Though common methods of immobilization on surfaces rely on mercaptopropionic acid (MPA) or 3-aminopropyltriethoxy-silane (APS) followed by activation with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) [6,11], we used a much simpler method adapted from silica-silane chemistry. Since a passivated titanium surface exhibits similar surface chemistry to that of silica [12], a bifunctional linker, glycidoxypropyltrimethoxysilane (GOPTS), was used as the linking molecule [13,14].

GOPTS presents, on one end, a trialkyloxysilane group, like APS, which allows its incorporation on activated silica or silica-like surfaces. Once GOPTS is deposited on a surface, it exposes epoxy moieties which are unstable and therefore very reactive toward nucleophilic groups such as amines, thiols, and alcohols [14]. These groups will promptly react causing opening of the bond angle strained epoxide ring. The main advantage of GOPTS over other bifunctional linkers is that it does not require an activation step, whereas activation with EDC/NHS is required for standard carboxylic functionalities. In our work, GOPTS' trimethoxysilane end was bound to the titanium surface of the microcantilever and CSA-1 antibody was subsequently immobilized on the sensor under basic conditions, through addition of its amine groups to epoxide rings exposed on the surface. Figure 3, details the steps that take
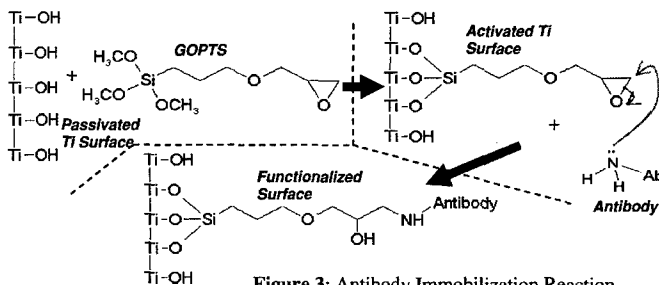


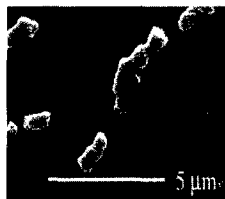Figure 3: Antibody Immobilization Reaction Schematic



Figure 4: Scanning electron micrograph of S. typhimurium adhered to Ti surface

place during this reaction.

Following the functionalization of the microcantilever with antibody, several detections of the *S. typhimurium* cells were performed. In order to perform detection, the cantilever tip was lowered into a vial containing the cell suspension by means of a micropositioner such that approximately two thirds of the tip was submerged. This allowed for the monitoring of the resonant frequency shift with time as described above.

## RESULTS AND DISCUSSION

Before performing cell detection experiments, a verification that indeed cells were adhering to the sensor tip surface was needed. It was deduced, that if indeed cells were adhering to the surface, then the immobilization of the antibody by means of the GOPTS linker must necessarily also have been successful. Figure 4, above, is an SEM micrograph taken of *S. typhimurium* cells adhered to a titanium surface upon which the CSA-1 antibody had been immobilized, thereby confirming the antibody functionalization of the titania. The surface in this image was first treated with GOPTS as detailed above, then exposed to the antibody solution in borate buffer (pH = 9), then rinsed with PBS, then exposed to the salmonella cell suspension, and then rinsed again with PBS such that only cells firmly adhered to the surface would remain after the final rinsing.

In the current detection setup, the titanium tip was only partially immersed in the solution to avoid wetting of the PZT layer by the solution. As a result, there was an upward background resonance shift due to receding water level along with the resonance frequency shift due to cell binding. As a result, a typical cell-detection resonance frequency shift versus time exhibited an initial decrease, reaching a minimum that was followed by an almost linear rise as shown by the solid curve in Fig. 5a. To address the issue of background resonance frequency shift, we did three different checks. The first was to obtain the rate of the upward background shift in water prior to the detection experiment. The second was to obtain the rate of the upward background shift using the later stage of the resonance frequency shift that showed an almost linear increase. The third check was to use a two-cantilever array to perform the detection. One cantilever was treated with the GOPTS as detailed above, while the other cantilever was left passivated, but untreated with
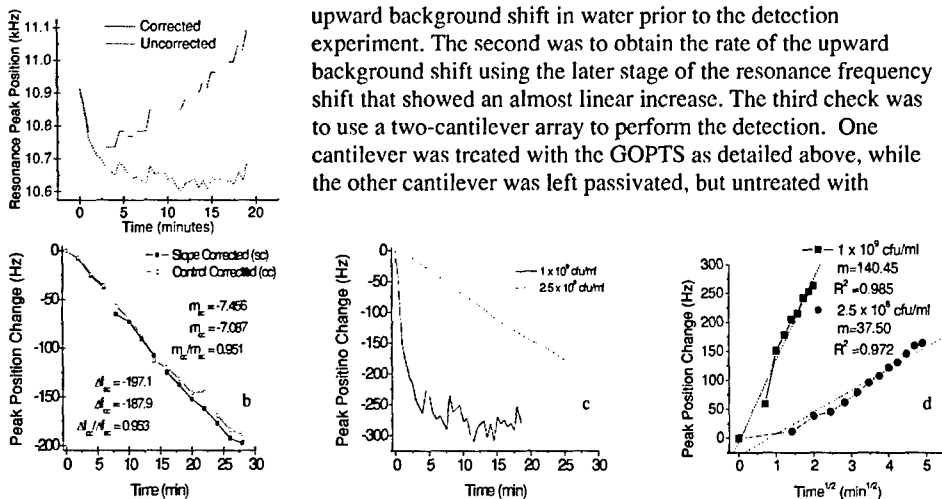
**Figure 5:** (a) Uncorrected salmonella detection data with corresponding corrected data. (b) Comparison of slope corrected vs. control corrected detection. (c) Detection of $1 \times 10^9$ and $2.5 \times 10^8$ cfu/ml S. typhimurium suspensions (d) Kinetic analysis of S. typhimurium binding

GOPTS. Then, both cantilevers were simultaneously subjected to the same series of steps associated with a detection process: (1) exposure to the antibody (2) rinse in phosphate buffered saline (PBS) and (3) dipping in the cell suspension. We found the two first corrections were practically the same, indicative that the upward shift in the later stage of detection was indeed a manifest of receding water level. In Fig. 5a, the corrected resonance frequency shift due to cell binding is shown as the dashed line after subtracting the background upshift from the initial data (the solid line). Figure 5b, shows two curves from the same detection of a $2.5 \times 10^8$ cfu/ml *S. typhimurium* suspension. In this figure, the *control corrected* data represents the subtraction of the upward drift based on the control cantilever that was not coated with the GOPTS (and thus was not functionalized with the CSA-1 antibody). The *slope corrected* data represents the removal of the linear drift simply by taking a linear regression of the detection data after cell adhesion on the sensor tip had ceased. As can be seen in the figure, the ratio of the two slopes is nearly unity as is the ratio of the total resonant frequency shifts. Furthermore, as mentioned above, the cantilever used Figure 5a was also monitored in water alone and the same linear up-drift was obtained, thus providing further substantiation of the post-cell adhesion up-drift subtraction method. Based on these findings, all subsequent cell detection experiments used only the post-cell adhesion linear drift data to correct for the upward drift. This greatly reduced the complexity of the experiments and thus allowed for more efficient data collection.

In addition to the detection of this $2.5 \times 10^8$ cfu/ml suspension, a detection of a $1 \times 10^9$ cfu/ml suspension was performed and these results were compared. Figure 5c displays these two curves; from this plot, we can see that the higher concentration detection experiment saturates the cantilever tip surface at approximately 5 minutes, whereas the lower concentration experiment only begins to yield saturation near 30 minutes. Taking the analysis of these data a step further, a comparison of the adhesion kinetics associated with the two concentrations was performed. It can be shown that for high concentrations, such as we have here, the concentration of the solution is linearly proportional to the square of the detection time by an adsorbtion per unit area factor [15]:

$$\frac{\Gamma}{c} \propto \sqrt{t} \qquad (2)$$

where $\Gamma$ is the adsorption per unit area factor, $c$ is the concentration of the cell suspension, and $t$ is the detection time. Plotting the same data from Figure 5c in Figure 5d on the $\sqrt{t}$ scale, we can see that both lines are quite linear and that the ratio of the slopes is similar to that of the concentrations: The ratio of the concentrations is 0.250 while the ratio of the slopes is 0.267. This clearly indicated that by carefully monitoring the detection kinetics, not only the presence of the cells is detected but the concentration of the cells can be quantified.

Using an estimated weight for a single Salmonella cell along with a tip surface coverage factor determined by scanning electron microscopy, the detection sensitivity for the cantilever can be calculated to be $1 \times 10^{-10}$ g/Hz. This number relates the mass adhered to the tip that will generate 1 Hz of frequency shift. Thus, for this cantilever, each nanogram of mass adhered to tip surface will generate 10 Hz of frequency shift which is easily discernable using the impedance monitoring technique. This is already a great degree of sensitivity, further miniaturization will yield even greater sensitivity – calculation has shown that femtogram sensitivity can be reached by reducing the cantilever length to 0.1 mm [9]

## CONCLUSIONS

It has been shown that the PZT-Ti microcantilever is an effective tool for direct, in-situ detection and quantification of *Salmonella typhimurium* in solution. By using a unique protein immobilization procedure that allows for the functionalization of titanium, the immobilization of CSA-1 antibody on the sensor tip surface was confirmed and successful dose response detections of *S. typhimurium* were performed using the PZT-Ti microcantilever. A detection sensitivity of 0.1 ng/Hz was achieved and further miniaturization promises still greater sensitivity. As opposed to present techniques used in industry, this method of detection demonstrates real-time, *in situ* detection and quantification as well as lending itself to the development of an array system such that multiple detections may be performed at once. In addition, the all-electrical actuation and detection makes the sensor and measuring unit light-weight and portable.

## ACKNOWLEDGEMENTS

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